

*CELLS INVOLVED IN THE IMMUNE RESPONSE, IX.  
DEPLETION FROM THE NORMAL RABBIT BONE  
MARROW OF ANTIGEN-REACTIVE CELLS DIRECTED  
TOWARD HUMAN PERIPHERAL LEUKOCYTES\**

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*Abstract.*—Normal adult rabbits were injected intravenously with peripheral leukocytes obtained from a normal human volunteer (donor A). The animals were sacrificed 24 hours later, and their bone marrow cells (primed bone marrow) or bone marrow cells obtained from a normal, unimmunized rabbit (normal bone marrow) were injected intravenously into irradiated (800 r) rabbits. The latter were immunized with peripheral leukocytes of donor A. The antisera obtained from the irradiated rabbits given primed bone marrow cells displayed minimal or no cytotoxic activity toward white cells of either donor A or an unrelated donor B. On the other hand, antisera obtained from irradiated rabbits given normal bone marrow cells displayed high titers of cytotoxic activity with respect to donor A cells only. It is concluded that rabbit bone marrow can be depleted of antigen-reactive cells directed toward white cell antigens following injection of the white cells intravenously and that these bone marrow cells are incapable of conferring antibody-forming capacity, in irradiated recipients, directed to this particular species of white cells. The relevance of these findings to the field of transplantation in general is discussed.

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The role of the bone marrow as a prime source of immunocompetent cells has been suspect for a number of years, but it is only recently that experimental evidence has been presented which implicates the bone marrow in the cellular processes concerned with the induction and mediation of the primary immune response. It has been demonstrated that the antibody-forming cell in the mouse,<sup>1</sup> the cell mediating the cellular or delayed immune reaction in the rat,<sup>2, 3</sup> and the antigen-reactive cell in the rabbit<sup>4, 5</sup> are all of bone marrow or hematogenous origin. These cells in the rabbit normally inhabit the bone marrow but vacate the marrow immediately following contact with the antigen *in vivo*.<sup>4, 5</sup> Current investigations in the laboratory have, in fact, suggested that the bone marrow in the rabbit serves as the prime, if not the only, source of antigen-reactive cells in the rabbit.<sup>6</sup> Bone marrow cells obtained from an animal immunized 24 hours previously (primed bone marrow) are incapable of mediating an immune response in an irradiated recipient toward the antigen used to immunize the donor.<sup>5</sup> However, these cells can mediate an immune response to all other antigens in a recipient irradiated rabbit.<sup>5</sup> Since it has been shown that the antibody-forming cell in an irradiated rabbit that had been injected with normal allogeneic bone marrow is of host, and not donor origin,<sup>7</sup> it must be con-

cluded that the transferred bone marrow cells contained only antigen-reactive cells and no antibody-forming cells.

In view of the observation that bone marrow can be specifically depleted of antigen-reactive cells directed to a particular antigen, thus rendering it immunoincompetent with respect to this antigen, it was felt that the system could be applied in a practical way to the field of transplantation. Since it has been observed that normal bone marrow can be rendered immunoincompetent with respect to protein antigens, such as human serum albumin, and to cellular antigens, such as sheep red blood cells, after the administration of these antigens, it was felt that the human white cell might act in exactly the same manner following its injection into the rabbit. As demonstrated below, normal rabbit bone marrow can be depleted of antigen-reactive cells directed to human peripheral lymphocytes.

*Materials and Methods.*—Human peripheral leukocytes were obtained by bleeding normal human volunteers via the antecubital vein with a heparinized syringe. The blood was transferred to sterile plastic tubes (Falcon Plastics, Los Angeles, Calif.) which were placed at a 60° angle to the horizontal in a 37°C incubator. After 30 to 45 min, the tubes were placed in an upright position, and the white cell-containing supernatants were drawn off and transferred to other sterile tubes. The cells were centrifuged at 600 rpm for 10 min and suspended in Medium 199 (Microbiological Associates, Bethesda, Md.) in a concentration of  $10^8$  cells/ml. The cells ( $10^9$ ) were injected intravenously into normal adult New Zealand white rabbits (Fig. 1) which were killed 24 hr later by intravenous injection of Nembutal (50 mg/kg body wt). The femur and tibia were cleaned and split with a bone cutter, and the bone marrow (primed bone marrow) was transferred to sterile plastic tubes, each of which contained 5 ml sterile normal rabbit serum (Microbiological Associates, Bethesda, Md.). The tubes were shaken for several minutes in order to free the cells from the marrow matrix and centrifuged at 500 rpm for 10 min. The cells were suspended in Medium 199 and centrifuged a second time. They were then resuspended in this medium in a concentration of  $10^8$  cells/ml. Bone marrow cells were obtained from normal, unimmunized rabbits in an identical fashion. The cells were injected intravenously into rabbits which had just been subjected to 800 r total body irradiation from a Cobalt 60 source (Fig. 1). The animals were also injected intravenously with  $2.5 \times 10^8$  human peripheral white cells (Hu-wbc) obtained from the same donor whose cells were used to prime the rabbits (donor A).

The immunized rabbits were bled at various intervals of time thereafter, and the antisera were tested for antibody activity with respect to the human wbc. The following procedures were used: (a) *Determination of cytotoxic activity directed toward human leukocytes by the dye-exclusion test.*<sup>8</sup> Aliquots (0.5 ml) of the antisera, or various dilutions of the antisera, were incubated with  $4 \times 10^6$  Hu-wbc for 1 hr at 37°C. Guinea pig complement, 0.25 ml, was then added to each tube, and the latter were incubated for another hour at 37°C. One drop of a 2% solution of trypan blue was then added to each tube, and the cells were examined under the microscope to determine the number of dead cells, which permitted penetration of the dye. The results are presented as percentages of dead cells of the entire cell population. A total of 200 cells was counted and the results were extrapolated to the original cell suspension. (b) *Inhibition of blastogenesis by phytohemagglutinin* The human peripheral leukocytes were suspended in Medium 199, containing 10–15% autologous plasma, 100 units penicillin, and 100 µg streptomycin, to a concentration of  $10^6$  cells/ml. Aliquots (4 ml) were transferred to sterile disposable plastic tubes (Falcon) to which were added 0.25-ml aliquots of the rabbit anti-Hu-wbc antisera and 0.25 ml complement. The tubes were capped and incubated at 37°C for 1 hr. The cells were then washed twice with Medium 199 and resuspended in this medium containing 20% fetal calf serum. Phytohemagglutinin (diluted 10-fold, 0.25 ml) (Difco Lab-

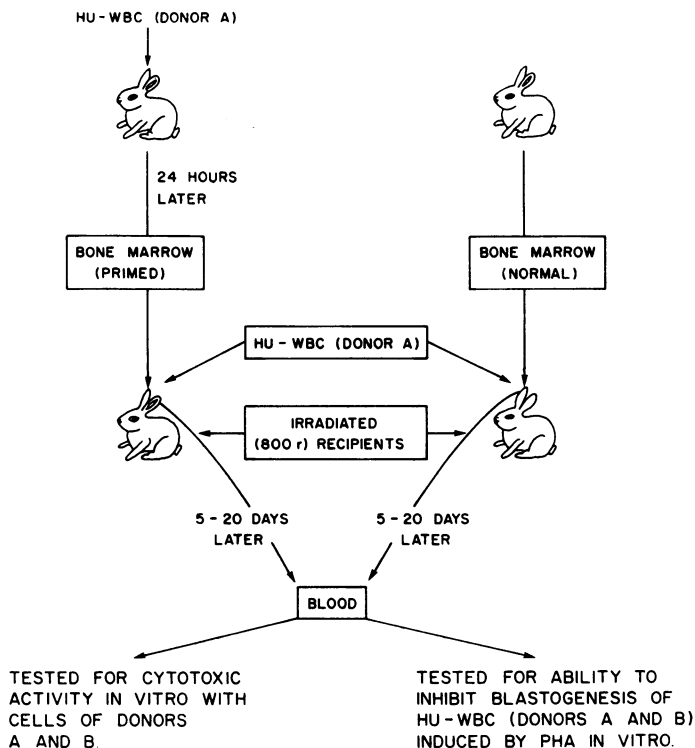


FIG. 1.—Experimental protocol. Depletion from rabbit bone marrow of cells immunologically reactive toward human peripheral white blood cells (HU-WBC).

oratories, Detroit, Michigan) was added to the specified tubes, and the tubes were sealed and incubated at 37°C for 3 days. Tritiated thymidine (2  $\mu$ Ci, sp. act. 1 Ci/mM) was added on day 2. At the termination of culture, the tubes were centrifuged at 2000 rpm for 10 min and resuspended in 2 ml of a 5% solution of trichloroacetic acid. They were centrifuged a second time, resuspended in the trichloroacetic acid, and centrifuged once more. To each tube was added  $\frac{1}{2}$  ml of Hyamine 10  $\times$  (Packard Instruments, Downers Grove, Ill.), and the cells were permitted to digest overnight. The contents of the tubes were then transferred to counting vials with 0.6 ml of absolute ethanol to which were added 15 ml of scintillation solution. The radioactive content of the tubes was determined with a Packard Model 4000 liquid scintillation counter. The procedure outlined above has previously been described in detail.<sup>4</sup>

The leukocyte culture ("two-way stimulation") technique, as described originally by Bain *et al.*,<sup>9</sup> was used for the demonstration of antigenic nonidentity of the peripheral leukocytes of the two human donors (donors A and B). Briefly, the peripheral leukocytes, obtained and prepared as described above, were suspended in Medium 199 containing penicillin, streptomycin, and autologous plasma, in a concentration of  $10^6$  cells/ml. Two ml aliquots of the cell suspension of donor A cells were mixed with an equal volume of cells of donor B in sterile disposable plastic (Falcon) tubes which were then tightly capped and maintained in a 37°C incubator for 7 days. Two  $\mu$ Ci tritiated thymidine (sp. act. 1 Ci/mM) were added to each tube on day 6 of culture. At the termination of culture, the cells in each tube were processed for their radioactive content in the manner described above.

In several cases, the "one-way stimulation" technique, as described by Bach and Voynow,<sup>10</sup> was utilized. Here, one of the cell species was preferentially inactivated by

incubation with mitomycin C ( $25 \text{ gamma}/2 \times 10^6$  cells) for 20 min and then washed three times in Medium 199 prior to their addition to the tubes containing the cells of the other donor. In this variation of the mixed leukocyte culture technique, the only cells capable of undergoing blastogenesis are the cells which had not been inactivated by the mitomycin C. These tubes were incubated for 7 days at  $37^\circ\text{C}$  and treated in the same manner as described above for the "two-way stimulation" test.

**Results.**—The anti-human leukocyte antiserum prepared in irradiated rabbits that had been injected with "primed" allogeneic bone marrow cells displayed cytotoxic activity to a degree only slightly greater than that observed with the control, preimmunized isologous serum (Table 1). On the other hand, antiserum raised in irradiated rabbits given normal allogeneic bone marrow cells produced antisera with cytotoxic activity greatly exceeding that observed with the isologous control serum. Approximately 40 to 50 per cent of the leukocytes of the original human donor were killed upon incubation with these latter rabbit antisera, whereas only 2 per cent of the cells were killed after incubation with the sera of the same rabbits obtained prior to incubation. The antisera attained maximum cytotoxic capacity by day 12 after immunization.

TABLE 1. *Cytotoxic activity of rabbit antisera.*

| Antiserum from<br>rabbit given:* | Prior to<br>immunization<br>(undiluted serum) | Cytotoxicity (Percentage of Dead Cells)† of Antisera Obtained:<br>—On Day 12 after Immunization— |                            |                            |
|----------------------------------|---|--|----------------------------|----------------------------|
|                                  |   | Undiluted<br>serum   | Serum<br>(diluted<br>10 ×) | Serum<br>(diluted<br>40 ×) |
| Primed bone marrow‡              | 2   | 4  | 0                          | 0                          |
| Normal bone marrow               | 2   | 54   | 35                         | 5                          |

\* Rabbits were subjected to 800 r total body irradiation followed by the intravenous administration of allogeneic bone marrow ( $300 \times 10^6$  cells) (either normal or primed) and  $2.5 \times 10^8$  Hu-wbc.

† Donor peripheral leukocytes were incubated with the rabbit serum, complement, and trypan blue (2% solution).

‡ Bone marrow obtained from rabbit 24 hr after intravenous administration of  $10^9$  Hu-wbc.

When the antisera were tested for cytotoxicity in various dilutions, it was observed that antiserum produced in recipients of normal bone marrow cells had to be diluted more than 40-fold in order to bring the cytotoxic index down to that observed with the control serum, whereas the antisera induced in recipients of primed bone marrow cells possessed slight cytotoxic activity detected only if the serum were tested in the undiluted state (Table 1).

The specificity of the anti-wbc antiserum produced was then tested with respect to its reactivity toward the human wbc used for immunization and toward white cells obtained from an unrelated individual. The nonidentical nature of these two white cell suspensions was demonstrated by using the mixed leukocyte culture technique. The leukocytes of the original donor (donor A) responded well, with blastogenesis and mitosis, when incubated with mitomycin-C-treated wbc (one-way stimulation) obtained from the unrelated donor (donor B) (1200 cpm per culture tube). In the absence of an allogeneic stimulus, the original donor cells incorporated much less tritiated thymidine in culture (170 cpm per culture tube). The high incorporation of tritiated thymidine by the cells of donors A and B cultured together, when neither was inactivated by mitomycin C (17,500 cpm per culture tube), attests to the validity of the assumption that

the two species of leukocytes are genetically and/or antigenically dissimilar. When the cells of these two donors were incubated with the rabbit anti-donor *A* wbc antiserum and complement, only donor *A* cells were killed by the antiserum (50% dead cells), whereas the cytotoxic activity of the antiserum was negligible toward cells of donor *B*.

Further evidence substantiating the immunoincompetence of primed bone marrow is presented in Table 2. Incubation of anti-donor *A* wbc antiserum, obtained from a recipient of normal bone marrow, with donor *A* leukocytes and complement for one hour resulted in the subsequent inhibition of blastogenesis of these cells by phytohemagglutinin. The antiserum, even in a 160-fold dilution, was still capable of inactivating donor *A* leukocytes to the extent that they could not respond optimally to subsequent stimulation with phytohemagglutinin (specific incorporation of only 0.69) (Table 2). On the other hand, blastogenesis of donor *A* cells by phytohemagglutinin was not affected after incubation of donor *A* leukocytes with complement and anti-donor *A* leukocyte antiserum obtained from a recipient injected with primed bone marrow cells and immunized with donor *A* wbc (Table 2).

TABLE 2. *Inhibition of PHA-induced blastogenesis of human peripheral lymphocytes.\**

| Antiserum obtained from rabbit given:†          | Cells incubated 1 hr with antiserum in presence of complement | Incorporation of tritiated thymidine (cpm/culture tube) in cells incubated 3 days with PHA | Specific incorporation‡ of tritiated thymidine by incubated cells |
|---|---|--|---|
| Normal bone marrow (pre-immunization)           | Undiluted   | 29,932   | —   |
| Normal bone marrow (day 19 after immunization)  | Undiluted   | 4,667  | 0.15  |
|   | 10 × dilution   | 6,228  | 0.21  |
|   | 40 × dilution   | 9,213  | 0.32  |
|   | 160 × dilution  | 20,839   | 0.69  |
| Primed bone marrow (pre-immunization)§          | Undiluted   | 24,038   | —   |
| Primed bone marrow (day 19 after immunization)§ | Undiluted   | 16,891   | 0.70  |
|   | 10 × dilution   | 24,779   | 1.00  |
|   | 40 × dilution   | 25,002   | 1.00  |
|   | 160 × dilution  | 24,914   | 1.00  |

PHA = phytohemagglutinin.

\* By prior incubation of these cells with rabbit anti-human leukocyte antiserum and complement.

† The rabbit was subjected to 800 r total body irradiation followed by the intravenous administration of either normal or primed allogeneic bone marrow cells ( $300 \times 10^6$  cells) and  $2.5 \times 10^6$  Hu-wbc.

‡ The specific incorporation of tritiated thymidine is defined as the ratio of thymidine uptake following incubation of the cells with the postimmunization antiserum to that incorporated after incubation of the cells with the preimmunization serum (control serum).

§ Bone marrow obtained from rabbit 24 hr after intravenous administration of  $10^6$  Hu-wbc.

If we omitted complement from the system during the initial incubation of the leukocytes with the antiserum, no subsequent inhibition of blastogenesis occurred, thus demonstrating the necessary presence of complement for the cytotoxic effect of the antiserum to be realized.

*Discussion.*—The present investigation was undertaken as a result of two apparently unrelated observations in the fields of cellular immunology and transplantation. The first is the recent finding that the immune response, in both the rabbit and the mouse, involves the interaction of the antigen with at

least two functionally and anatomically distinct but morphologically identical lymphoid cells, the antigen-reactive cell and the antibody-forming cell. In the rabbit, although the source of the antibody-forming cell is not yet known, the antigen-reactive cell is of bone marrow origin.<sup>5, 6</sup> In the mouse, recent findings by Mitchell and Miller<sup>1</sup> and Taylor<sup>11</sup> have considerably clarified the picture and it appears that the thymus serves as the source of antigen-reactive cells and the bone marrow as the store of antibody-forming cells or their precursors. The second observation, that in the field of transplantation, is that present attempts at suppressing the graft-rejecting mechanisms through the judicious use of antilymphocyte serum, steroids, antimetabolites, and other forms of therapy have not produced the anticipated results. Drug therapy does not appear to provide the answer to the problem. We therefore felt that new approaches to the problem of limiting host reactivity to homografts should be investigated, and the findings alluded to above indicated the direction to be taken.

It has been demonstrated that the administration of protein antigens, such as human serum albumin,<sup>4</sup> and cellular antigen, such as sheep erythrocytes,<sup>5</sup> results in the immunoincompetence of the bone marrow (in cell transfer experiments to irradiated recipients) with respect to these antigens. It has also been demonstrated that the antibody-forming cell in the irradiated recipient, which had received normal allogeneic bone marrow cells and antigen, is of host origin and not donor origin.<sup>7</sup> Therefore, it was concluded that the immunocompetent cell present in the bone marrow is the antigen-reactive cell and that the irradiated recipient still possesses his entire complement of antibody-forming cells, which are irradiation-resistant.<sup>7</sup> It was therefore postulated that bone marrow might also be rendered immunoincompetent with respect to its reactivity toward human transplantation antigens in a similar way. Since the circulating leukocytes in man possess transplantation antigens,<sup>12-18</sup> we decided to use human peripheral leukocytes (donor *A*) in initial attempts to deplete the bone marrow in the rabbit of antigen-reactive cells directed toward all the major antigens on the human white cell (primed rabbit bone marrow). If our concept is correct, then antiserum produced in the irradiated recipient rabbit given primed rabbit bone marrow and human donor *A* white cells should possess no antibodies directed toward donor *A* leukocytes, whereas antiserum produced in irradiated rabbits given normal allogeneic bone marrow should possess antibody activity directed toward donor *A* leukocyte but not to unrelated human donor *B* leukocytes. The results obtained were exactly as predicted and completely confirm our previous findings with other antigens.<sup>4, 5</sup>

The implication of these findings should be obvious to those investigators engaged in searching for ways to specifically suppress the graft-rejecting mechanism. Experiments using this system are currently under way, in the rabbit, to determine whether the transfer of primed bone marrow to irradiated allogeneic recipients permits a skin graft from the original cell donor to "take" on this recipient for a period longer than that observed with skin grafts from a rabbit unrelated to either the original white cell donor or the irradiated host. The outcome of these experiments should help to pave the way for an entirely new approach to be utilized in attempts to specifically inhibit the graft-rejection

mechanism without in any way incapacitating the immunological activities of the recipient toward other antigens.

A question that might be raised with respect to the interpretation of these and previously obtained results<sup>4, 5</sup> is whether the antigen-reactive cells actually vacate the bone marrow following interaction with the antigen *in vivo* or whether they become "tolerant" and persist in the bone marrow. Recent evidence supports the interpretation that the cells migrate out of the marrow since it has been shown that bone marrow cells can be incubated with the antigen *in vitro*, after which these cells can transfer antibody-forming capacity to this antigen in an irradiated recipient.<sup>6</sup> It has also been demonstrated that the bone marrow antigen-reactive cells can be retained if passed through an antigen-sensitized glass bead column and that they can subsequently be eluted from the column and shown to be capable of transferring antibody-forming capacity to the antigen in an irradiated recipient.<sup>19</sup> These findings demonstrate that interaction of the antigen-reactive cell with the antigen does not result in immunological tolerance of the cells to the antigen.

The experimentally induced situation which permits one to obtain an apparently normal rabbit bone marrow cell suspension that is, however, devoid of antigen-reactive cells directed toward a single antigen or a group of antigens situated on the white cell and is therefore incapable of transferring antibody-forming capacity with respect to these white cells in an irradiated host has also been duplicated in the mouse. Taylor<sup>11</sup> has observed that primed mouse thymus cells lose the capacity to transfer immunocompetence if given along with normal bone marrow cells to irradiated recipients, whereas a mixture of normal thymus cells and normal bone marrow cells or normal thymus cells and primed bone marrow cells can transfer immunocompetence toward the specific antigen. Whether the situation present in the rabbit or the mouse can be extrapolated to man must be considered as pure conjecture at the present time. No evidence has as yet been presented to demonstrate whether the immune apparatus in man can be so neatly broken down into the antigen-reactive cell component and the antibody-forming cell component. Certainly, clinical findings in patients with various disorders concerned with immunoglobulin synthesis (dysgammaglobulinemias) have indicated a bursal influence as well as a thymic role in the maturation of the immunocompetent cells and their responsiveness to stimulation by antigens.<sup>20, 21</sup> However, these data do not elucidate whether a mono- or pluricell interaction is involved in the induction of the immune response. In the rabbit and the mouse, the immune response has been successfully dissected into the antigen-reactive and antibody-forming components. Whether similar cell types exist in man and whether the organ-origin of these cells is, anatomically, of the rabbit type or the mouse type remains to be determined.

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<sup>1</sup> Mitchell, G. F., and J. F. A. P. Miller, *J. Exptl. Med.*, **128**, 821 (1968).

<sup>2</sup> Lubaroff, D. M., and B. M. Waksman, *J. Exptl. Med.*, **128**, 1425 (1968).

<sup>3</sup> *Ibid.*, p. 1437.

- <sup>4</sup> Singhal, S. K., and M. Richter, *J. Exptl. Med.*, **128**, 1099 (1968).
- <sup>5</sup> Abdou, N. I., and M. Richter, *J. Exptl. Med.*, **129**, 757 (1969).
- <sup>6</sup> *Ibid.*, in press.
- <sup>7</sup> Richter, M., and N. I. Abdou, *J. Exptl. Med.*, **129**, 1261 (1969).
- <sup>8</sup> Engelfreit, C. P., and A. Britten, *Vox Sanguinis*, **10**, 660 (1965).
- <sup>9</sup> Bain, B., M. R. Vas, and L. Lowenstein, *Blood*, **23**, 108 (1964).
- <sup>10</sup> Bach, F. H., and N. K. Voynow, *Science*, **153**, 545 (1966).
- <sup>11</sup> Taylor, R. B., *Nature*, **220**, 611 (1968).
- <sup>12</sup> Brent, L., *Progr. Allergy*, **5**, 271 (1958).
- <sup>13</sup> Snell, G. D., *Ann. Rev. Microbiol.*, **11**, 439 (1959).
- <sup>14</sup> Merrill, J. P., *Physiol. Rev.*, **39**, 860 (1959).
- <sup>15</sup> Lawrence, H. S., *Physiol. Rev.*, **39**, 811 (1959).
- <sup>16</sup> Snyder, G. B., P. A. Ebert, and M. T. Edgerton, *Bull. Johns Hopkins Hosp.*, **116**, 95 (1965).
- <sup>17</sup> Halasz, N. A., L. N. Seifert, H. A. Rosenfield, M. J. Orloff, and H. A. Stier, *Proc. Soc. Exptl. Biol. Med.*, **123**, 924 (1966).
- <sup>18</sup> Abeyounis, C. J., F. Milgrom, and E. Witebsky, *Nature*, **203**, 313 (1964).
- <sup>19</sup> Abdou, N. I. and M. Richter, *J. Exptl. Med.*, **130**, 141 (1969).
- <sup>20</sup> Seligman, M., H. H. Fudenberg, and R. A. Good, *Am. J. Med.*, **45**, 817 (1968).
- <sup>21</sup> Cooper, M. D., A. E. Gabrielsen, and R. A. Good, *Ann. Rev. Med.*, **18**, 113 (1967).